Kinetics of $^{13}$N-ammonia uptake in myocardial single cells indicating potential limitations in its applicability as a marker of myocardial blood flow

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ABSTRACT To study kinetics and principles of cellular uptake of $^{13}$N-ammonia, a marker of coronary perfusion in myocardial scintigraphy, heart muscle cells of adult rats were isolated by perfusion with collagenase and hyaluronidase. Net uptake of $^{13}$N, measured by flow dialysis, reached equilibrium within 20 sec in the presence of sodium bicarbonate and carbon dioxide ($pH$ 7.4, $37^\circ$ C). Total extraction, 80 sec after the reaction start, was 786 ± 159 $\mu$mol/ml cell volume. Cells destroyed by calcium overload were unable to extract $^{13}$N-ammonia. Omission of bicarbonate and carbon dioxide reduced total extraction to 36% of control. $^{13}$N-Ammonia uptake could also be reduced by 50 $\mu$M 4,4' diisothiocyanostilbene 2,2' disulfonic acid, by 100 $\mu$g/ml 1-methionine sulfoximine, and by preincubation with 5 $\mu$M free oleic acid. These results indicate that in addition to metabolic trapping by glutamine synthetase, the extraction of $^{13}$N-ammonia by myocardial cells is influenced by cell membrane integrity, intracellular-extracellular pH gradient, and possibly an anion exchange system for bicarbonate. For this reason, the uptake of $^{13}$N-ammonia may not always provide a valid measurement of myocardial perfusion.


$^{13}$N-ammonia ($^{13}$N),* a positron-emitting radionuclide, has been used as a marker of myocardial perfusion.1–8 Because cellular extraction of an ideal flow marker must be proportional to regional myocardial blood flow, extraction of such a marker should not be influenced by cell metabolism or cell membrane transport mechanisms. Since myocardial extraction fraction of $^{13}$N-ammonia can be significantly reduced when intracellular glutamine synthetase is inhibited,6,7 the usefulness of this compound as a flow marker has been questioned. This study provides additional data regarding the kinetics and possible role of the plasma membrane in myocardial $^{13}$N uptake and retention in isolated adult rat heart cells.

Materials and methods

Preparation of myocardial single cells. Male Wistar rats 3 to 4 months old were heparinized and anesthetized by urethane (1.5 mg/g body weight, intraperitoneally). Hearts were extracted and perfused in a nonrecycling manner for 10 min with 35 mM NaCl, 4.75 mM KCl, 1.2 mM KH$_2$PO$_4$, 150 mM sucrose, 25 mM NaHCO$_3$, 10 mM HEPES buffer, 10 mM glucose, and 5 g/liter albumin bovine fraction V ($pH$ 7.4, $37^\circ$ C, 95% O$_2$/5% CO$_2$).9 A recycling perfusion (25 min) was carried out with the same solution also containing 150 U/ml unpurified collagenase (E.C.3.4.4.19.) and 1000 U/ml hyaluronidase (E.C.3.2.1.36.). At the end of the perfusion the softened heart was sliced into small pieces and reincubated for 10 min in the same enzyme-containing solution; the suspension was filtered through a nylon mesh and centrifuged gently for 2 min at 50 g. The supernatant was removed and the pellet was washed three times in 130 mM NaCl, 4.0 mM KCl, 1.2 mM KH$_2$PO$_4$, 25 mM HEPES buffer, 10 mM glucose, and 20 g/liter albumin bovine fraction V ($pH$ 7.4, room temperature, 100% O$_2$). A small population of completely destroyed cells in these cell suspensions could be easily distinguished from cells with a morphologically intact structure by light microscopy. Intact cells were typically rod shaped, cross-striated, and sometimes showed spontaneous irregular contractions. A stepwise return to physiologic concentrations of calcium was possible after the cells were suspended for 15 min in 100 mM KCl, 30 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 20 mM taurine, 5.5 mM ATP, 3.0 mM pyruvate, 5.0 mM succinate,
During the reaction only the free, rapid, exchangeable part of the glycocalyx as described by Isenberg et al., i.e., they were destroyed by calcium overload.

Nonviable cells were prepared by incubation of cells in a reaction medium containing 3.5 mM CaCl₂ immediately after perfusion with collagenase and hyaluronidase. The separated cells prepared in this manner showed the typical "calcium paradox", i.e., they were destroyed by calcium overload.

Preparation of electron micrographs. Isolated heart muscle cells were fixed in 2.5% glutaraldehyde containing 0.3M NaCl and 5 g/liter albumin bovine fraction V (pH 7.4), the reaction suspension gave a final concentration of 100 mM of total calcium (0.1, 0.5, 2.0, and 3.5 mM).

These cell suspensions contained about 65% to 80% intact cells that did not contract spontaneously; however, rhythmic contractions could be induced by the external application of current impulses. Electron micrographs revealed alterations of the glycocalyx as described by Isenberg et al., but other ultrastructural features were not abnormal.

For intervention studies the suspension, containing calcium tolerant cells, was diluted to 1.5 ml, slowly mixed, and divided into two equal parts. Both parts were gently centrifuged (2 min at 50 g) and the supernatants were discarded. One pellet was always resuspended in 0.75 ml of medium I (control); the other was resuspended in 0.75 ml medium II or in medium I containing either 56 μM 4,4’ diisothiocyanostilbene-2,2’ disulfonic acid (DIDS), 111 μg/ml 1-methionine-sulfoximine, or 5.5 μM oleic acid (no albumin). The oleic acid used was suspended in the reaction medium by intense sonication. All studies were carried out with lead citrate and uranylacetate and examined with a Zeiss EM 10 microscope.

Reaction media. Two different reaction media, medium I and medium II, were used in the measurements of 13N uptake. Medium I contained 110 mM NaCl, 4.75 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 20 mM HEPES buffer, 25 mM NaHCO₃, 10 mM glucose, 5 g/liter fatty acid-free bovine albumin, and 3.5 mM CaCl₂. This medium was equilibrated by 5% CO₂ and 95% O₂ at pH 7.4. Medium II, gassed with 100% O₂, had all the components of medium I except NaHCO₃, and the NaCl concentration was 135 mM (pH 7.4).

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Measurement and calculation of 13N uptake. Cellular extraction of 13N-ammonium was measured by a flow dialysis system as described by Colowick et al. and shown schematically in figure 1. The reaction chamber of the flow dialysis apparatus contained 0.45 ml of myocardial cells. The reaction suspension, which was gently stirred with a magnetic stirrer, was separated from the flow chamber by a dialysis membrane permeable to substances under a molecular weight of 5000. During the experiment, reaction medium was pumped at a constant rate through a flow chamber and the gamma counter.

All reactions were started by the addition of 50 μl 13N-ammonium chloride, dissolved in medium I or II (pH 7.4, 37°C) so as to avoid changes in temperature, pH, and electrolyte concentration at the start of the reaction. Ammonium chloride added to the reaction suspension gave a final concentration of 100 μM. During the reaction only the free, rapid, exchangeable part of 13N was able to enter the flow chamber by diffusion through the dialysis membrane. The gamma counter automatically recorded the total counts of the effluent at 5 sec intervals. The concentration of 13N in the flow chamber (C₂) reached an equilibrium at a time (t) that depended on flow rate (f = 12.8 ml/min), total volume of the flow chamber plus that of the tube leading to the gamma counter (V = 0.90 ml), the membrane constant D, and the concentration of the diffusible part of 13N-ammonium chloride in the reaction chamber (C₁) according to the following equation:

\[ C₂ = C₁D/(1 - e^{-dt/f}) \]

The decrease of the total amount of 13N in the reaction chamber caused by diffusion into the flow chamber during the experiment was below 0.5% and could be neglected. To get a standard count rate per mol 13N, the count rates of three aliquots of the reaction suspension corrected for physical decay were measured after each extraction and related to the total amount of 13N in the reaction chamber.

From the above equation, the membrane constant D was obtained by duplicate calibration measurements carried out without cells in the reaction medium. Figure 2 gives an example of the time course of 13N activity in the flow chamber, corrected for physical decay. Because 20 sec were required before equilibrium was reached in the flow chamber, no interventions were made during the uptake reaction. To estimate the amount of extracted 13N at 5, 10, and 15 sec, the corresponding "apparent" membrane constants, as obtained from the calibration curves, were used. The apparent initial rate of 13N uptake was determined from the slope given by the 5 and 10 sec values.

After determination of D and calculation of the count rate per mole 13N as described above, the freely diffusible part of 13N in the reaction chamber was calculated from 13N activity measured in the flow chamber. From the difference between the amount of freely diffusible 13N in the absence and presence of cells in the reaction medium, the amount of 13N extracted by myocardial cells was calculated for each time interval, and then related to total cell volume that was estimated by calculation of (1H)-water and (14C)-inulin space in the cell suspension of the control and intervention phases as described by Powell et al. 14H and 14C were measured in a liquid scintillation counter (Tri-Carb 4500; Packard Instruments, Frankfurt, F.R.G.), with count rates corrected for background and channel overlap.
Materials. $^{13}$N-Ammonia was produced in a cyclotron, according to the $^{16}$O(P,$\alpha$)$^{13}$N reaction.$^{15}$ The following materials were obtained from Sigma Chemie GmbH, Taufkirchen, F.R.G.: disodium salt of DIDS, 1-methionine sulfoximine, bovine serum albumin fraction V, sodium salt of oleic acid, and fatty acid-free bovine serum albumin (fraction V). Hyaluronidase was obtained from Serva Feinbiochemica, Heidelberg, and collagenase type CLS1 No. 498245P was purchased from Seromed GmbH, München, F.R.G.

Myocardial cells were stimulated by a SD-5C stimulator (Grass Medical Instruments, Quincy, MA). Dialysis membranes were purchased from Kalle, Hoechst, F.R.G. (pore diameter 2.5 to 8.0 nm).

To measure $^{13}$N activity, a gamma counter from Berthold, Wildbad, F.R.G. (H.V. supply BF 2302, ratemeter LB 2232, scaler timer BF 2270-1, interface BF 2222 TEL) and a NaI(Tl) crystal were used (type 7SF8/G; Harshaw Chemie B.V., The Netherlands).

Results

All data obtained in this study are summarized in table 1. As described in Materials and methods, cell preparations were contaminated by irreversibly destroyed cells (table 1), which could not be removed without significantly reducing the total amount of intact myocardial cells. For this reason the role of unspecific binding of $^{13}$N by nonviable cells was determined. There was no difference between the calibration curve without cells and the curve obtained by suspending destroyed cells in the reaction medium (figure 2, left). Dead cells therefore did not extract measurable amounts of $^{13}$N.

The cellular extraction of $^{13}$N by myocytes under control conditions (medium I, 95% O$_2$, 5% CO$_2$, pH 7.4, 37°C) was rapid, reaching an equilibrium within 15 sec (figure 3). The apparent initial rate of $^{13}$N net uptake was reduced to 9% of the control value when cells were suspended in medium II, in which sodium bicarbonate was replaced by HEPES buffer and carbon dioxide was omitted. Total uptake, 80 sec after the reaction started, was 36% of the control value.

Since the dependence of $^{13}$N uptake on the presence of bicarbonate and carbon dioxide could be explained if cellular $^{13}$N uptake were mediated by an anion exchange system, the effect of DIDS was investigated. As shown in figure 4, 50 μM DIDS depressed total

<table>
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<td>$^{13}$N uptake in myocardial single cells</td>
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<tr>
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<th>Apparent initial rate of $^{13}$N uptake (pmol/cm$^2$sec)$^A$</th>
<th>Total $^{13}$N uptake (nmol/ml)</th>
<th>Fraction of nonviable cells (%)$^B$</th>
<th>No. of experiments</th>
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<tr>
<td>Control</td>
<td>140 ± 55$^C$</td>
<td>786 ± 159$^C$</td>
<td>25.1 ± 7.4 (26.9 ± 7.0)$^C$</td>
<td>21</td>
</tr>
<tr>
<td>Medium II</td>
<td>12 ± 8</td>
<td>285 ± 80</td>
<td>25.0 ± 5.9 (26.7 ± 6.6)</td>
<td>6</td>
</tr>
<tr>
<td>Medium I + 50 μM DIDS</td>
<td>37 ± 28</td>
<td>280 ± 60</td>
<td>27.3 ± 7.5 (27.6 ± 8.1)</td>
<td>6</td>
</tr>
<tr>
<td>Medium I + 100 μM DIDS</td>
<td>48 ± 29</td>
<td>330 ± 110</td>
<td>20.2 ± 5.7 (22.5 ± 5.3)</td>
<td>4</td>
</tr>
<tr>
<td>1-methionine sulfoximine</td>
<td>18 ± 14</td>
<td>180 ± 88</td>
<td>27.8 ± 5.3 (29.8 ± 6.9)</td>
<td>5</td>
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$^A$For calculation of the apparent initial rate, a surface area of 530 cm$^2$/g myocardium was assumed.$^{16, 17}$

$^B$Fractions of nonviable cells before and after the observation period (in parentheses) in percent of the total cell amount.

$^C$Values are mean ± SD.
FIGURE 3. Calculated net uptake of $^{13}$N in medium I under control conditions (●) and in medium II when sodium bicarbonate was replaced by HEPES buffer and CO$_2$ was omitted (○). Values are mean ± SD (n = 6).

up take to 35% of the control value. A similar effect was seen in 50 µM, 4,4′dinitrostilbene2,2′disulfonic acid (data not shown).

Low concentrations of oleic acid (figure 5) reduced the apparent initial rate to 13% and total uptake to 23% of the control value. As demonstrated by Bergmann et al. and Schelbert et al., who measured $^{13}$N uptake and washout in the whole heart of rabbits and dogs, respectively, $^{13}$N uptake could be depressed by l-methionine sulfoximine, an inhibitor of intracellular glutamine synthetase. In the presence of 100 µg/ml l-methionine sulfoximine, apparent initial rate and total uptake were reduced to 34% and 42% of the control values, respectively (figure 6). The cells remained morphologically intact in the presence of DIDS, oleic acid, and l-methionine sulfoximine, and both tolerated extracellular calcium and responded with ordered contractions to electrical stimuli.

FIGURE 4. Influence of DIDS on cellular $^{13}$N uptake in medium I (gassed with 5% CO$_2$ and 95% O$_2$). ● = control; ○ = preincubation with 50 µM DIDS. Values are mean ± SD (n = 6).

FIGURE 5. Time course of cellular $^{13}$N accumulation in the absence (●) and presence of 5 µM free oleic acid (○). Experiments were carried out with a slightly modified reaction medium (medium I, no albumin), as described in the text. Cells were preincubated in the oleic acid containing medium I 30 min before the reaction was started. Values are mean ± SD (n = 5).

Discussion

$^{13}$N-Ammonia has been used as a flow marker in myocardial scintigraphy, but the ability of this tracer to quantify regional myocardial blood flow has been questioned because $^{13}$N uptake and retention depend on metabolic trapping as well as on myocardial perfusion. The results of this study indicate that cellular uptake of $^{13}$N also depends on the integrity of the plasmalemmal membrane and possibly on an anion exchange system that can be inhibited by stilbene disulfonic acids.

Single heart muscle cells prepared from adult rats were used to eliminate influences of regional blood flow, blood vessel wall, and interstitial space. Apart from alterations of the external lamina of the glycoalyx, the cells were morphologically intact as shown by

FIGURE 6. Cellular $^{13}$N uptake in the absence (●) and presence of 100 µg/ml l-methionine sulfoximine (○). Experimental conditions are described in Materials and methods. Values are mean ± SD (n = 4).
electron microscopy. The cells were also calcium tolerant and showed rhythmic contractions only after the application of current impulses. As demonstrated by metabolic and electrophysiologic studies, these features provide strong criteria for cell viability, and loss of the external lamina of the glycocalyx does not interfere with major functions of the cell membrane such as the slow inward current, electromechanical coupling, activation of adenylate cyclase, and insulin-stimulated glucose transfer. Although dead cells could seriously interfere with the results by binding \(^{13}\)N nonspecifically, our results demonstrated that dead cells did not extract significant amounts of \(^{13}\)N.

The flow dialysis system used in this study allowed the uptake of the short-lived \(^{13}\)N isotope to be measured and largely avoided mechanical alterations of the cells during the experiment. However, the flow dialysis system is suitable only if cellular extraction of the tracer is sufficiently high to reduce markedly the extracellular concentration during the experiment. Because there was a delay of 20 sec until the flow system reached equilibrium (figure 2), the membrane constants calculated from calibration measurements at 5, 10, and 15 sec were only apparent, so that the calculations based on these apparent membrane constants can give only approximations of the real uptake velocity.

The calculated amount of \(^{13}\)N extracted by myocardial cells does not represent the concentration of ammonium chloride in the cytosol because the amount of intracellular ammonium chloride present before the start of the reaction was not taken into account in this study. Furthermore, a significant amount of the accumulated \(^{13}\)N was metabolized by the glutamine synthetase pathway (figure 7, step 7), as evidenced by the marked reduction of total \(^{13}\)N uptake by l-methionine sulfoximine, a known glutamine synthetase inhibitor. A similar reduction of \(^{13}\)N uptake by l-methionine sulfoximine has been observed in perfused rabbit and dog hearts. Additional pathways, such as the urea cycle, could also metabolize \(^{13}\)N.

The uptake and retention of \(^{13}\)N could also be influenced by changes in the translocation through the sarcolemma since \(^{13}\)N participates in an acid-base equilibrium between ammonium ions and NH\(_3\). The ammonium ion is unlikely to cross the lipid bilayer by simple diffusion, but this ion can serve as a potassium analog in the activation of the sarcolemmal Na-K-ATPase. Since extracellular ammonium concentrations in the present study were well below the K\(_m\) of the complex of ammonium with the Na-K-ATPase, studies of the ion selectivity of potassium channels showed a permeability ratio between ammonium and potassium ions of only 0.13.

Lipid-soluble NH\(_3\), however, is known to cross the lipid bilayer rapidly and, and the association of protons with intracellular NH\(_3\) is also very rapid (figure 7, step 1). Because plasma membrane permeability to NH\(_4^+\) is lower than that to NH\(_3\), the distribution of NH\(_4^+\) across the sarcolemma will be determined largely by the intracellular-extracellular pH gradient, as shown in studies of the distribution of ammonia between blood and cerebrospinal fluid.

Intracellular pH has been reported to be 0.2 to 0.5 units lower than extracellular pH. This would result in an intracellular-extracellular concentration ratio of NH\(_4^+\) of 1.6 to 3.2, provided intracellular alkalization induced by NH\(_3\) influx is compensated for by a buffer system. Intracellular pH could be maintained by an anion exchange system as it has been described in red blood cells and in sheep cardiac Purkinje fibers (figure 7, step 6). The ability of stilbene disulfonic acids and the absence of carbon dioxide and bicarbonate to inhibit \(^{13}\)N uptake suggests that an anion exchange system is present in the sarcolemma of rat myocytes. Stilbene disulfonic acids such as DIDS and DNDS are known to be specific inhibitors of anion exchange systems in erythrocytes. Carbon dioxide plays an important role in regulating intracellular pH, so that if CO\(_2\) rapidly crosses the plasma membrane and is metabolized within the cell to H\(^+\) and HCO\(_3^-\) via the carbonic anhydrase reaction (figure 7, step 5), the reduction of intracellular buffer capacity in the absence of CO\(_2\) could decrease cellular \(^{13}\)N uptake. Dependence of ammonia uptake on CO\(_2\) has been demonstrated earlier in skeletal muscle and in eryth-

**FIGURE 7.** Proposed reaction scheme of \(^{13}\)N uptake into the myocardial cell. GS = glutamine synthetase; Glu = glutamate; Gln = glutamin; i = intracellular space; o = extracellular space; CA = carbonic anhydrase. See text for details.
cytes. Because the intracellular-extracellular pH gradient has been reported to be reduced in hypercapnic acidosis, the moderate reduction of 13N extraction when plasma pH is lowered (Schelbert et al.) may reflect the putative participation of the intracellular-extracellular pH gradient in the regulation of 13N uptake.

A number of studies suggest that changes in lipid metabolism may lead to altered cardiac function, and free fatty acids have been shown to modify the Na-K-ATPase, the calcium pump ATPase of sarcoplasmic reticulum, and cell membrane fragility. This study extends these observations with the finding that oleic acid reduces 13N uptake. Since the oleic acid concentrations used in this study neither caused morphologic alterations of the cells nor changed extracellular pH, the reduction of 13N uptake could be explained by a functional change in the cell membranes such as inhibition of the postulated anion exchange system or a change in membrane permeability leading to an increase in 13N efflux. However, further studies are needed to evaluate these and other possible mechanisms of this effect of oleic acid.

The data presented in this report indicate that 13N-ammonia may not be a valid marker for regional myocardial blood flow under all conditions because 13N-ammonia uptake depends not only on perfusion but also on the viability of the myocardial cells, metabolic factors, and the function of the plasma membrane. Since ammonia is metabolized by glutamine synthetase in a reaction that requires ATP, the extent of ammonia metabolism may depend on the energetic state of the myocardial cell. Thus the reduction of intracellular ATP to concentrations in the range of the Km for the enzyme-ATP complex, which is about 1.5 mM in rat skeletal muscle, could reduce intracellular 13N metabolism by glutamine synthetase and therefore inhibit 13N extraction. As demonstrated by the effect of oleic acid, changes in lipid metabolism could also influence the capability of the myocardial cell to accumulate 13N-ammonia. Furthermore, changes of the intracellular-extracellular pH gradient caused by intracellular and extracellular acidosis during ischemia or by dysfunction of the postulated anion exchange system could influence 13N uptake. These factors may complicate the interpretation of clinical myocardial blood flow measurements by 13N-ammonia scintigraphy under different pathologic conditions.

We thank W. Winnweisser and H. Gasper for their excellent technical assistance and Dr. Arnold M. Katz for his help in preparing this manuscript.

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Circulation. 1985;71:387-393
doi: 10.1161/01.CIR.71.2.387

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